PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Albert DURANTON et al. Group Art Unit: 1611

Application No.: 10/517,423 Examiner: G. YU

Filed: March 10, 2005 Docket No.: 122005

For: USE OF TAURINE FOR TREATING ALOPECIA

DECLARATION UNDER 37 C.F.R. §1.132

I, Duranton Albert, a citizen of France, hereby declare and state:

1. I have a degree in DVM Doc IIIième cycle which was conferred upon me by Ecole Nationale Vétètinaire & Institut Polytechnique Paul Sabatier in Toulouse in 1985.

2. I have been employed by L'Oreal since 1986 and I have had a total of 20 years of work and research experience in treatment methods for hair follicle miniaturization and alopecia.

3. I am a member of Ordre National de Vétèrinaires

4. My publications include the following works in this field: Collin C, Gautier B, Gaillard O, Hallegot P, Chabane S, Bastien P, Peyron M, Bouleau M, Thibaut S, Pruche F, Duranton A, Bernard BA. Protective effects of taurine on human hair follicle grown in vitro. Int J Cosmet Sci. 2006 Aug;28(4):289-98

5. I am a named inventor in the above-captioned patent application.

6. I am not being directly compensated for my work in connection with this Declaration.

7. I and/or those under my direct supervision and control have conducted the following tests :

Abbreviations

TGF-β: Transforming Growth Factor β
CPM: Counts Per Minute
DMEM: Dulbecco's modified Eagle's medium
Esm: Standard error of the mean
NHDF: Normal Human Dermal Fibroblast
Sd: Standard deviation
FCS: Fetal Calf Serum
MW: Molecular Weight

Materials

Cells and media

-Cell type: NHDF; reference Bio*alternatives*, batch PF2 (used at the 8th passage).

-Culture conditions: 37°C, 5 % CO_2

-Culture medium: DMEM completed with L-glutamine 2mM, Penicilline 50 U/ml, Streptomycine 50 µg/ml, FCS 10 %

-Test medium: DMEM completed with L-glutamine 2mM, Penicilline 50 U/ml, Streptomycine 50 µg/ml, FCS 1 %

Tested compounds

-Green tea polyphenols (MW: 458.4 $g.mol^{-1}$), stock solution concentration: 100 mM in DMSO.

-Taurine (MW: 125.15 $g.mol^{-1}$), stock solution concentration: 100 mM in ultrapure water.

-TGF-β: stock solution concentration: 250 ng/ml

General description

Taurine alone, polyphenols alone and the combination of polyphenols and taurine were tested on NDHF, wherein proline-rich proteins (namely collagens) hyperproduction was induced by adding TGF-β.

The effect of the tested components was evaluated through the measure of radiolabelled proline (i.e. L-[2,3-^{3}H]-proline) incorporation.

L-[2,3-^{3}H]-proline incorporation measure

Cells were cultivated in a culture medium for 24 hours. At confluence, the medium was replaced by test medium completed with TGF-β at 1 ng/ml, with or without ("blank") the components to be tested (taurine and polyphenols, separately, and their combination). Cells were then incubated for 72 hours. In order to study proline-rich protein hyperproduction, radio-labelled marker L-[2,3-^{3}H]-proline (333 µCi/ml) was added for the last 24 hours of culture. All the experimental conditions were conducted in triplicate.

Proline-rich protein hyperproduction is characterized by the accumulation of both secreted proline-rich proteins and "constitutive" proline-rich proteins (i.e. intercellular/matrix proteins).

Consequently, L-[2,3-^{3}H]-proline incorporation was evaluated both in secreted proline-rich proteins (i.e. soluble proteins) and, analogous to the test performed in the application as filed (see pages 25-26), in neosynthesized proline-rich proteins (i.e. intercellular/matrix proteins) in the fibroblasts.

Thus, at the end of incubation, supernatents were collected on the one hand and "cell tissues" were lysed by adding chaotropic buffer on the other hand.

In both case, proteins were precipitated with trichloroacetic acid and recollected on filter and L-[2,3-^{3}H]-proline incorporation remaining on the support was evaluated by liquid scintillation.

In the following tests, the results are expressed as a percentage of the control (i.e. with TGF-β only).

More particularly, the greater the decrease in percentage of detected radioactivity, the more proline incorporation is affected and thus the more hyperproduction of proline-rich proteins is inhibited.

PART I: EFFECT OF POLYPHENOLS AND TAURINE SEPARATELY

i) Effect on secreted proline-rich proteins (supernatent)

All the results are reported in the following table 1.

Table 1

Tested components	Concentration	cpm	cpm mean	esm	% Control	esm (%)	Statistic significance treshold (p)
Control (TGF-β)	1 ng/ml	4099 3475 3436	3670	215	**100**	6	-
Green tea polyphénols	10 µM	3220 2779 3109	3036	132	**83**	4	ns
	100 µM	1899 1806 1396	1700	155	**46**	4	**
Taurine	1 mM	4635 3226 3480	3780	434	**103**	12	ns
	5 mM	3922 3340 3378	3547	188	**97**	5	ns

Ns: $p>0.05$: Non significant
*: $0.01 \leq p \leq 0.05$: Significant
**: $0.001 \leq p \leq 0.01$: Very significant
***: $p<0.001$: Extremely significant

As expected, after TGF-β treatment, the secretion of proline-rich proteins was increased. This result validates the assay.

Concerning polyphenols alone, at 10 µM, no significant effect was observed on proline incorporation.

At a higher concentration (100 µM), proline incorporation was significantly affected.

However, at this concentration, an important cytotoxic effect has been revealed by microscopic observations.

Concerning taurine alone, at 1 or 5 mM, proline incorporation was not significantly affected.

ii) Effect on intercellular/matrix proline-rich proteins

All the results are reported in the following table 2.

Table 2

Tested components	Concentration	cpm	cpm mean	esm	% Control	esm (%)	Statistic significance treshold (p)
Control (TGF-β)	1 ng/ml	4257 3448 4817	4174	397	**100**	10	-
Green tea polyphénols	10 µM	2453 3230 3398	3027	291	**73**	7	ns
	100 µM	1091 1108 1305	1168	69	**28**	2	**
Taurine	1 mM	2648 3165 4066	3293	414	**79**	10	ns
	5 mM	2184 3200 3385	2923	373	**70**	9	ns

Ns: $p>0.05$: Non significant
*: $0.01\leq p\leq 0.05$: Significant
**: $0.001\leq p\leq 0.01$: Very significant
***: $p<0.001$: Extremely significant

As expected, after TGF-β treatment, the synthesis of proline-rich proteins by the fibroblasts was increased. This result validates the assay.

Concerning green tea polyphenols, the results are similar to those obtained on soluble proteins (same tested concentrations).

Concerning taurine, at 1 mM and 5 mM, proline incorporation in intercellular/matrix proline-rich proteins was not significantly modified.

PART II: EFFECT OF POLYPHENOLS/TAURINE COMBINATION

i) Effect on secreted proline-rich proteins (supernatent)

This assay was performed as previously described in Part I and the corresponding results are summarized in the following table 3.

Table 3

Tested components	Concentration	cpm	cpm mean	esm	% Control	esm (%)	Statistic significance treshold (p)
Control (TGF-β)	1 ng/ml	4167 4121 4000	4096	50	100	1	-
Green tea polyphénols + Taurine	1 µM + 1 mM	2418 2598 2305	2440	85	60	2	***

Ns: $p>0.05$: Non significant
*: $0.01\leq p\leq 0.05$: Significant
**: $0.001\leq p\leq 0.01$: Very significant
***: $p<0.001$: Extremely significant

As expected, after TGF-β treatment, the secretion of proline-rich proteins was increased. This result validates the assay.

The green tea polyphenols/taurine combination at 1 µM/1 mM significantly affected proline incorporation.

ii) Effect on intercellular/matrix proline-rich proteins

The assay was performed as previously described in Part I and the corresponding results are summarized in the following table 4.

Table 4

Tested components	Concentration	cpm	cpm mean	esm	% Control	esm (%)	Statistic significance treshold (p)
Control (TGF-β)	1 ng/ml	4382 4315 4276	4324	31	100	1	-
Green tea polyphenols + Taurine	1 µM + 1 mM	2461 2278 2856	2532	171	59	4	***
	10 µM + 5 mM	2969 2575 2562	2702	134	62	3	***

Ns: $p>0.05$: Non significant
*: $0.01 \leq p \leq 0.05$: Significant
**: $0;001 \leq p \leq 0.01$: Very significant
***: $p<0.001$: Extremely significant

As expected, after TGF-β treatment, the synthesis of proline-rich proteins by the fibroblasts was increased. This result validates the assay.

As for soluble proteins, the green tea polyphenols/taurine combination 1 µM/1 mM significantly affected proline incorporation.

Furthermore, such a combination comprising 10 µM of polyphenols and 5 mM of taurine also significantly affected proline incorporation.

CONCLUSION

Taurine alone, tested at 1mM or 5 mM, has no significant inhibition effect, either on secreted proline-rich proteins or on neosynthesized proline-rich proteins, i.e. intercellular/matrix proteins (see tables 1 and 2).

However, when 1 µM of polyphenols is added to the same concentration of 1mM of taurine, a significant inhibiting effect was observed on both secreted proline-rich proteins and neosynthesized proline-rich proteins, i.e. intercellular/matrix proteins (see tables 3 and 4),

whereas polyphenols alone, at a 10-time higher concentration (10μM) showed no significant inhibiting effect (see tables 1 and 2).

Furthermore, whereas polyphenols alone at 10 μM exhibited no inhibiting activity on neosynthesized proline-rich proteins (see table 2), a significant inhibition of the neosynthesized proline-rich proteins was observed when polyphenols, at the same concentration of 10 μM, was combined with 5 mM of taurine (see table 4).

In view of the foregoing, the combination of taurine and polyphenol thus has a synergistic and significant inhibiting effect on both secreted proline-rich proteins and on neosynthesized proline-rich proteins (i.e. intercellular/matrix proteins).

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: 18 2 2010 18/2/2010

DURANTON A.